fungal and radiographic studies were not thought to be necessary. A matrix biopsy was not performed because the patient and her family refused consent.

Comment. Samman[6] describes proximal nail loss without scarring as *periodic shedding* and categorized *autosomal dominant periodic nail shedding* as a subgroup of this category. Since 1897, there have been 4 case reports to our knowledge[2-5] of healthy adolescents or adults presenting with periodic shedding of their nails and a family history among several siblings or parents of the same patients. The first reported case of nonfamilial nail shedding was described in 2009 by Venugopal and Murrell.[7] Cases of idiopathic familial and sporadic onychomadesis are summarized in the **Table**.

The mechanism of nail matrix arrest in the setting of infection, fever, systemic disease, or drug exposure is unknown. Inhibition of cellular proliferation may occur and would be a logical explanation during treatment with antimitotic therapy. Another hypothesis is that the matrix activity and growth rate of the nails remains intact, but the quality of the manufactured nail plate differs, becoming thinner and dystrophic.

Idiopathic cases of onychomadesis can be classified as familial (autosomal dominant inheritance) or sporadic.

We describe herein a young, healthy female patient with no family history of onychomadesis and no seasonal variation in nail shedding. We suggest calling such unusual cases *idiopathic sporadic onychomadesis.*

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Perinatal Cytomegalovirus-Associated Bullae in an Immunocompetent Infant

Although perinatal cytomegalovirus (CMV) infection is common, symptomatic CMV infection is a rare complication in immunocompetent infants. We describe herein CMV-associated bullae accompanied by CMV hepatitis in an immunocompetent newborn infant is reported.

Report of a Case. The patient was a 1-month-old female infant of 39 weeks' gestation and vaginal delivery with no apparent complications. She presented with a history of crops of vesicles and bullae on the face, precordial area, and buttocks that began appearing at age 3 weeks and continued to appear. The lesions manifested as multiple 10-mm-diameter bullae with several crusts on the sites (**Figure 1** A and B).

Fluid content was purulent, and Tzanck test findings were negative. The patient had no fever, lymphadenopathy, or organomegaly, and her general condition and neonatal reflexes were normal. The findings of her neonatal screening test for metabolic diseases were negative. Birth weight was 2.99 kg, and she was normally breastfed.

A skin biopsy specimen from a newly formed blister in the skin overlying the lower jaw revealed severe spongiosis and reticular degeneration in the epidermis. Perivascular mixed inflammatory cell infiltration was seen, and the blood vessel wall was thickened in the superficial dermis (**Figure 2**). Cytomegalovirus was not detected by immunohistochemical staining of the skin specimen.

Laboratory tests demonstrated normal complete blood cell counts and normal biochemical findings except for levels of aspartate aminotransferase (AST) (406 U/L) and alanine aminotransferase (ALT) (325 U/L), suggesting active hepatitis (to convert AST and ALT to microkatal per liter, multiply by 0.0167). Serum concentration of immunoglobulin, complement titer, CD4/CD8 ratio, and lymphocyte stimulation test results were normal for her age. Serum anti-CMV IgM findings were negative, and anti-CMV IgG findings were positive at age 3 months. There was no serologic evidence of infection by herpes simplex virus (HSV), herpes zoster virus (HZV), or Epstein-Barr virus. Results of the pp65 antigenemia assay for circulating CMV antigen were positive (226 positive cells of 41 000 total cells). Real-time polymerase chain reaction (PCR) CMV findings were positive in urine and blood plasma samples. Cytomegalovirus DNA findings were negative on a Guthrie card, which meant that congenital CMV infection was excluded. No bacteria or HSV-1, HSV-2, or HZV specimens were isolated by cultures from the blister fluid. Findings of real-time PCR analyses for HSV and HZV sequences were negative for the blister fluid. The patient and her mother both tested negative for human immunodeficiency virus infection.

The bullae, hepatitis, and copy numbers of CMV were improved by anti-CMV hyperimmune gammaglobulin and intravenous ganciclovir treatment. The skin lesions healed with scarring (Figure 1C). Her physical and mental development was normal.

Comment. Congenital CMV infection or perinatal symptomatic CMV infection in immunocompromised or low-birth-weight infants occasionally shows systemic manifestations (eg, neurologic and developmental problems, hepatitis, pneumonia, retinitis, enterocolitis) and skin manifestations, including indurated

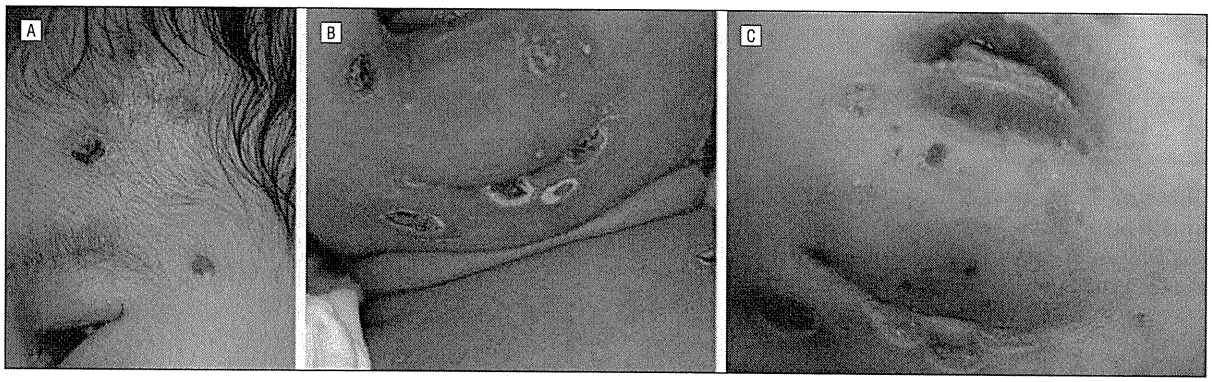

Figure 1. Skin manifestations of the patient. A and B, On the day of admission at patient age 1 month, pustules and erosions with crusts are scattered on the forehead (A) and on the chin and the chest (B). C, After administration of anticytomegalovirus agents, the skin lesions on the chin healed with scarring.

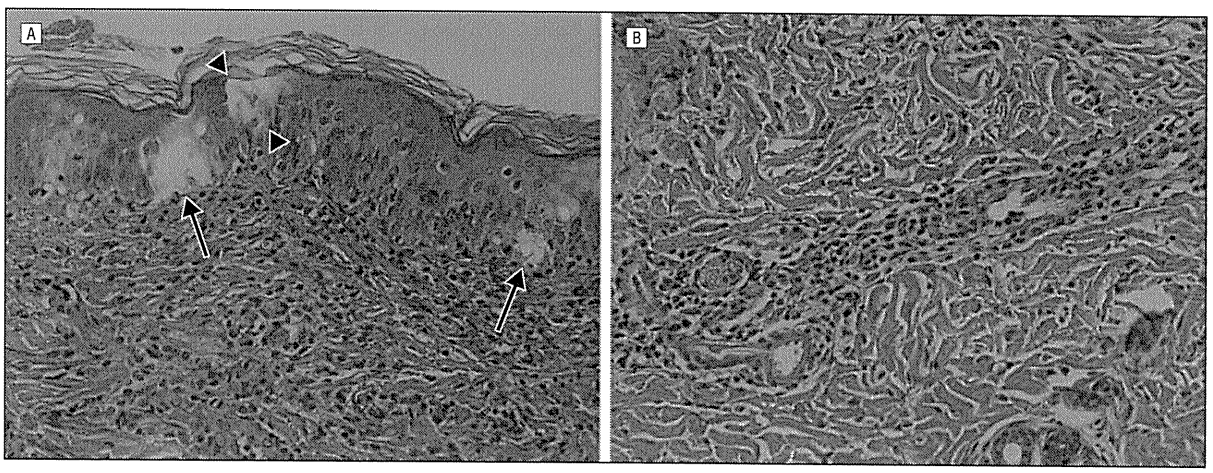

Figure 2. Histopathologic findings from the bulla on the lower jaw. A, Microblisters (arrows) and reticular degeneration (between triangles) of the epidermis are seen (hematoxylin-eosin, original magnification ×400). B, Perivascular inflammatory cell infiltration and thickening of the blood vessel wall are seen in the superficial dermis (hematoxylin-eosin, original magnification ×400).

hyperpigmented nodules and papules, papular and pruritic eruptions, vesiculobullous lesions, and perianal and perineal ulcers.[1-3] To our knowledge, symptomatic CMV-associated skin manifestations have not been previously reported in immunocompetent infants,[4] but CMV-associated erythema multiforme (EM) has been reported in immunocompetent adults.[5] The present case suggests that symptomatic CMV skin infection can occur not only in immunocompromised hosts and preterm infants but also in immunocompetent infants.

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Chinese Fortune-Telling Based on Face and Body Mole Positions: A Hidden Agenda Regarding Mole Removal

We were asked to remove moles from the faces of 2 Chinese children whose parents were born in mainland China. It was only after learning about their belief in Chinese mole reading did we understand their insistence. According to this belief, mole position, shape, size, and color have fortune-telling weight. The Thai of Chinese descent call this type of fortune-telling *Ngao Heng*.

Report of Cases. *Case 1*. A 12-year-old Chinese boy presented with an asymptomatic acquired nevus on his right nasolabial fold (**Figure 1**). Dermoscopy revealed a brown, bland-appearing nevocellular nevus with a globular pattern. The boy's father insisted on having the nevus removed even after he was assured that it was not necessary and the outlines of the scar were drawn on the boy's face.

Case 2. A 7-year-old Chinese girl (unrelated to the first patient) presented with a similar nevus on her cheek, just lateral to the nasolabial fold. Gross and dermoscopic examination revealed that the nevus was approximately 4 mm in diameter, bland in color, and normal in shape. The girl's mother insisted on removal. No amount of reassurance as to the benign nature of the nevus could dissuade her.

Comment. Chinese face and body mole reading is an ancient philosophy, recorded as early as 700 BC.[1] Other beliefs revolve around the shape and size of various facial parts. For example, if a person's ears stick out, they are called "wind-catching ears" and are equated to a self-serving personality. If a person has a prominent rounded nose, it means that he or she lacks support from other people. And if a person has a receding chin, it means that he or she will be poor.[1]

Chinese mole reading teaches that mole placement reflects on character and/or personality traits[2] (**Figure 2**). Mole placement can predict everything from good luck to bad luck, a healthy life vs a sickly life, success in business and marriage, and compatibility with people at home and work to the many fears, phobias, and wishes of everyday life.[3] Most people who follow Chinese mole reading check the "meaning" before making a decision about removal. Although removing the mole might not change the person's fate, it is often done to make the person feel better and to boost his or her confidence.

In our cases, the readings were "Tend to have problems related to diet or food," "Tend to have foot problems," and/or "Need to prevent water-related accidents." The philosophy of fortune-telling according to the location, color, and number of moles has been extensively written about and can be found in many sources. Currently, there are numerous Web sites for Chinese face and body mole predictions,[2,3] such as http://www.chinesefortunecalendar.com/FaceMoleReading.htm and http://www.weirdasianews.com/2010/01/11/chinese-face-readers-observe-moles/.

In conclusion, Asians brought up with Chinese physiognomy fortune-telling beliefs have dispersed all over the world. Many of them know and follow these

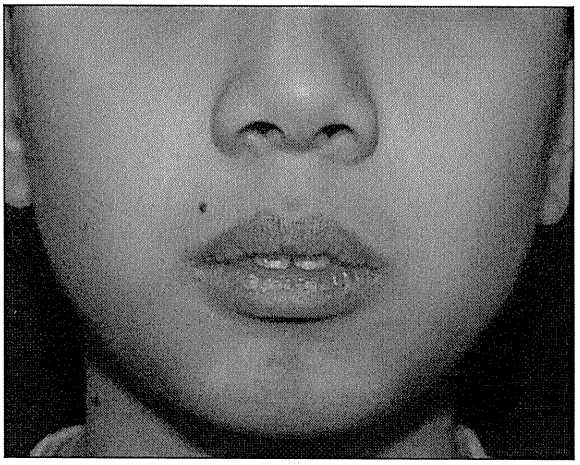

Figure 1. A 12-year-old Chinese boy with a nevocellular nevus on the right nasolabial fold.

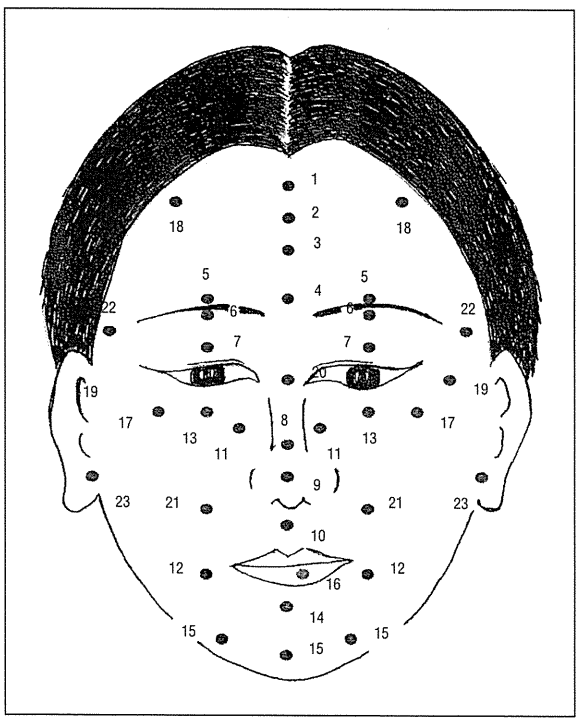

Figure 2. A facial mole–reading map (our own) based on http://www.chinesefortunecalendar.com/FaceMoleReading.htm. Our cases could be interpreted as "Tend to have problems related to diet or food," "Tend to have foot problems," and/or "Need to prevent water-related accidents."

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Concise report

Disappearance of anti-MDA-5 autoantibodies in clinically amyopathic DM/interstitial lung disease during disease remission

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Abstract

Objective. Autoantibodies against melanoma differentiation-associated gene 5 (MDA-5) are one of the serological markers for DM. Anti-MDA-5 antibodies are especially associated with rapidly progressive interstitial lung disease (ILD) in amyopathic DM (ADM). It is known that the antibody status of anti-ENAs does not generally change significantly with disease course. For anti-MDA-5 antibodies, however, few longitudinal studies have investigated such changes. This study aimed to establish a quantitative assay for anti-MDA-5 antibodies towards assessing the long-term outcome of ADM patients who had anti-MDA-5 antibodies.

Methods. We established ELISA for measuring anti-MDA-5 antibody levels using *in vitro* transcription and translation recombinant protein. The antibody levels were measured at different time points in 11 clinically ADM patients who tested positive for the anti-MDA-5 antibody on their first visit (range of follow-up 3 months to 16 years).

Results. At the stage of clinical remission, six patients received no medication and the four others received low-dose CS. ELISA showed that anti-MDA-5 antibodies disappeared in nine of the patients and fell to just above the cut-off in one patient; in the patient who died, the antibodies remained.

Conclusion. Our results suggest that anti-MDA-5 antibodies may be useful as a marker for monitoring disease activity in ILD complicated with ADM. Serial monitoring at short intervals is required to evaluate whether anti-MDA-5 antibody levels correlate with ADM disease activity.

Key words: amyopathic dermatomyositis, anti-MDA-5 antibody, interstitial lung disease, prognosis.

BASIC SCIENCE

Introduction

Myositis-specific autoantibodies are useful for diagnosing PM/DM. DM-specific autoantibodies against melanoma differentiation-associated gene 5 (MDA-5) and transcriptional intermediary factor 1-γ are particularly important, because they are closely associated with life-threatening complications such as rapidly progressive interstitial lung disease (ILD) and internal malignancies, respectively [1–4]. A subgroup of DM patients is known to have typical skin manifestations of DM but with little evidence of myositis, a condition known as clinically amyopathic DM (C-ADM). Initially, anti-MDA-5 antibodies were reported to be serological markers of clinically ADM with rapidly progressive ILD, especially in East Asia [5]; more recently they were found in Caucasian patients with ADM complicated with ILD [6]. Although it has been suggested that patients with anti-MDA-5 antibodies have a poor prognosis, few reports have tracked the long-term outcome of these patients [4, 7].

SLE is also an autoimmune rheumatic disease that is characterized by a fluctuating disease course and a variety of autoantibodies. Many autoantibody specificities (SSA/Ro, SSB/La, Sm, U1-RNP) in lupus patients remain constant over time, whereas reactivity to dsDNA may fluctuate with disease activity, although the pattern of change differs with autoantibody specificity [8, 9]. We have little information on an association between DM-specific

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autoantibodies and the long-term outcome of DM patients [10]. We established a quantitative assay of antibody levels and monitored anti-MDA-5 autoantibodies during long-term follow-up periods in order to assess the long-term outcome of ADM patients with anti-MDA-5 antibodies.

Materials and methods

Patients

The patients were seen or consulted in the Department of Dermatology, Nagoya University Graduate School of Medicine from 1994 to 2011. From our department serum bank, we used sera from 51 patients with DM, including 30 with C-ADM and 1 with C-ADM overlapping with scleroderma. These patients were diagnosed as having DM or C-ADM based on the criteria of Bohan *et al.* [11] and of Sontheimer [12], respectively. In general, C-ADM presents as typical skin lesions and amyopathy or hypomyopathy for >6 months. The ADM group included patients who developed fatal ILD within 6 months after disease onset. Of these 51 patients, 41 were characterized in our previous study [3]: 21 were anti-MDA-5 positive and 20 were negative. This study also included additional serum samples from 10 other DM patients with anti-MDA-5 antibodies, who were seen after our previous study [3] and defined by our immunoprecipitation assays with recombinant MDA-5. The anti-MDA-5-positive serum samples totalled 31 (male : female = 5 : 26). The mean age was 48.9 (range 11–80) years. One patient with JDM was included. Twenty sera were collected from healthy blood donors and used as normal controls.

In the 31 patients with anti-MDA-5 antibodies, sera from 10 patients with ADM were taken both at their first visit and at inactive disease periods after therapy. Serum from one other patient with ADM (female, aged 46 years) was taken at her first visit and just before death from ILD 3 months later. All the patients except one were female, and their ages ranged from 23 to 60 years. They were non-smokers and had no evidence of cancer. Ten of the patients developed ILD within 6 months after disease onset, whereas one patient had no lung involvement during the course. The first sera samples from all the patients were characterized as having had anti-MDA-5 antibodies previously [3]. The range of follow-up was 5–16 years, except for the patient who died. All the patients and healthy individuals in the present study gave fully informed consent for participation, including provision of sera samples. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and conducted in accordance with the Declaration of Helsinki.

ELISA

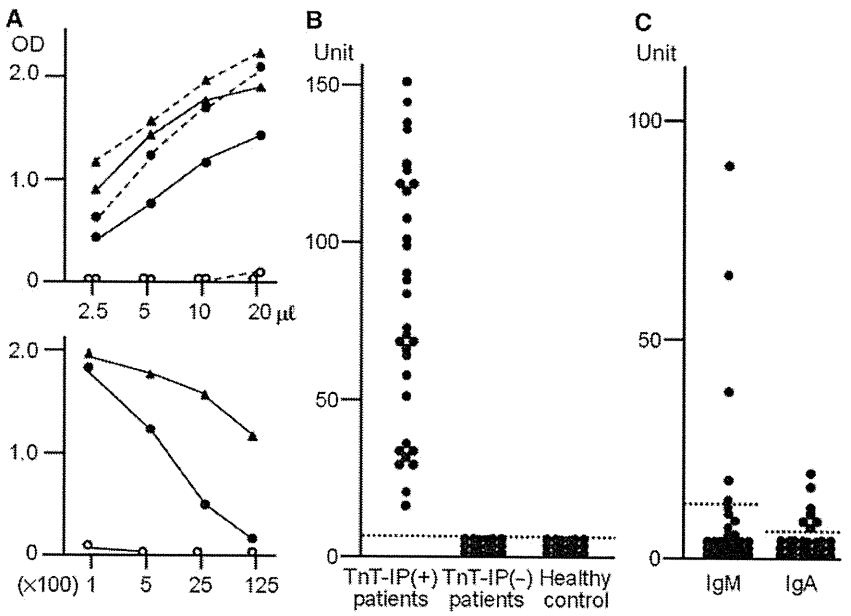
Specific binding of serum autoantibodies to recombinant MDA-5 was analysed using direct solid-phase ELISA. Biotinylated recombinant MDA-5 was produced from full-length MDA-5 cDNA using the TnT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) according to our protocol [3]. Nunc Immobilizer Streptavidin plates (Thermo Scientific Nunc, Roskilde, Denmark) to which streptavidin was covalently coupled via a spacer were pre-washed three times with PBS containing 0.05% Tween-20 (T-PBS) and were coated with biotinylated recombinant MDA-5 diluted with T-PBS (50 μl/well) and incubated for 1 h at room temperature. After three washes with T-PBS, the wells were blocked with 200 μl of a blocking buffer of 0.5% BSA (Wako, Osaka, Japan) in T-PBS for 1 h. Uncoated wells were used to measure the background levels for each sample. Diluted sample sera with blocking buffer (75 μl/well) were incubated for 1 h at room temperature, followed by incubation with anti-human IgG, IgM or IgA antibody conjugated with HRP (Dako, Glostrup, Denmark) as a secondary antibody (75 μl/well) at 1 : 30 000 dilution after five washes. After incubation for 1 h at room temperature, the plates were washed five times and incubated with Ultra TMB (Pierce, Rockford, IL, USA) (75 μl/well) as the substrate, according to the manufacturer's protocol. Then, optical density (OD) at 450 nm was determined using Multiskan FC (Thermo Scientific, Waltham, MA, USA). Each serum sample was tested in duplicate, and the mean OD subtracted background was used for data analysis. An in-house ELISA was used for measuring anti-diphtheria toxoid (DT). In brief, plates (Medisorp, Thermo Scientific Nunc) were coated with 50 μl/well DT (1 μg/ml in PBS) (List Biological Laboratories, Campbell, CA, USA) and blocked with 3% BSA in T-PBS. The sera samples were diluted 1 : 100 in 3% BSA in T-PBS. Anti-human IgG antibody conjugated with HRP and a substrate was used in the manner described above.

Results

ELISA with biotinylated recombinant MDA-5

To measure anti-MDA-5 antibodies in sera quantitatively, we tried to establish an ELISA that uses biotinylated recombinant MDA-5. Based on the results of two different anti-MDA-5-positive sera (Fig. 1A), we decided to use the 10 μl/well of TnT mixture and the diluted patient serum samples at 1 : 500 for measuring all samples. The unit of each sample was calculated as that sample's OD divided by the OD of the standard positive serum #1251 and then multiplied by 100. With the cut-off value determined as the mean value of 20 control sera + 3 S.D., 31 serum samples that had been identified as positive for anti-MDA-5 antibodies by immunoprecipitation also tested positive in these ELISA, and 20 serum samples from patients that were identified as being without anti-MDA-5 antibodies by immunoprecipitation also tested negative (Fig. 1B). We also measured IgM- and IgA-class antibodies using these assays as a positive control for the IgG anti-MDA-5 antibody level of #1251 (Fig. 1C). Both immunoglobulin classes of anti-MDA-5 antibodies were present, but in minor populations.

FIG. 1 ELISA using biotinylated recombinant MDA-5 protein. (**A**) Serial dilution of biotinylated protein (upper panel) or sera (lower panel) for ELISA. Closed circle and triangle: anti-MDA-5-positive sera in immunoprecipitation analysis. Open circle: healthy individual serum. In the upper panel, broken and solid lines denote sera diluted to 1:100 and 1:500, respectively. Recombinant protein was diluted with T-PBS to 50 μl of the final volume per well. In the lower panel, recombinant protein was applied at 10 μl diluted with 40 μl of T-PBS per well. Serum dilution was 1:100–1:12 500. (**B**) Measurement of anti-MDA-5 antibodies in 71 serum samples. All samples were classified as positive or negative for anti-MDA-5 antibodies, as determined by immunoprecipitation assay with biotinylated proteins. Broken line indicates the cut-off value (6.5 U), calculated from the mean OD values of 20 healthy controls + 3 S.D. (**C**) Isotype analysis of anti-MDA-5 antibodies. Thirty-one IgG anti-MDA-5-positive serum samples were also analysed for IgM and IgA class antibodies. Broken lines indicate the cut-off value (9.4 U for IgM and 6.0 U for IgA) calculated from the mean values of 20 healthy controls + 3 S.D.



Decline in anti-MDA-5 antibodies during remission

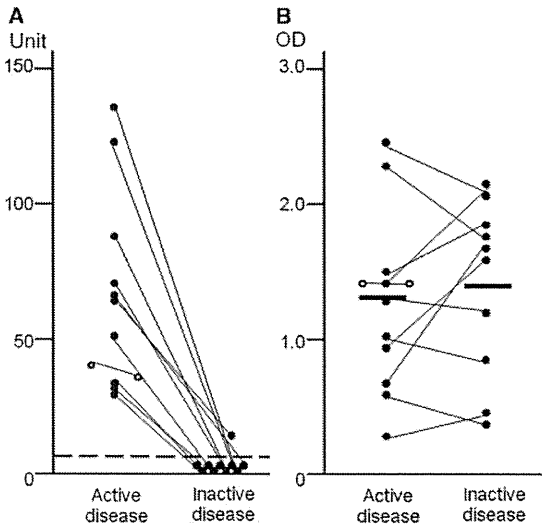
From 31 patients whose initial serum samples had anti-MDA-5 antibodies, sera were retaken during remission periods from the 10 patients with C-ADM. As a treatment for ILD in nine of these patients, methylprednisolone pulse therapy and immunosuppressive drugs were administered to eight patients and seven patients, respectively. The following immunosuppressive drugs were administered: ciclosporin to two patients, the combination of ciclosporin and i.v. CYC to two patients; ciclosporin, AZA and i.v. CYC to two patients and AZA and i.v. CYC to one patient. After initial therapy, 6 of the 10 patients were in clinical remission, which was defined as no evidence of active skin rash, myositis and lung involvement for >6 months without drug therapy. The remaining four patients also entered clinical remission, but with therapy of low-dose CS (prednisolone <7.5 mg/day). None of the 10 patients showed aggravated interstitial findings in their chest radiograph examinations for >5 years. The sampling of sera during remission ranged from 5 to 15 years after the first sampling. IgG-class anti-MDA-5 antibody levels were compared between serum samples at active and inactive disease states (Fig. 2A). Except for one patient who still had anti-MDA-5 antibodies but whose titre was dramatically reduced at 5 years from disease onset, in all the sera the anti-MDA-5 antibodies were absent during remission. These were also confirmed to be negative in the same ELISA plate with 20 sera samples from healthy individuals, and also by immunoprecipitation using this biotinylated protein (data not shown).

We also measured anti-DT antibodies in the same serum samples because we wondered whether the disappearance of anti-MDA-5 antibodies related to general immunosuppression. ELISA results showed that antibodies against DT remained at similar levels (Fig. 2B).

Discussion

In a Japanese multicentre study, 5-year survival in patients with anti-MDA-5 antibodies was 56% [4]. However, the long-term outcome of ADM patients has been seldom reported in terms of longitudinal serological findings. Since we recently examined >10 ADM patients

FIG. 2 Decrease of anti-MDA-5 antibody levels in remission. (**A**) Anti-MDA-5 antibody levels in 10 patients with ADM who were positive for serum anti-MDA-5 antibodies at their first visit decreased during inactive disease periods under the cut-off level (6.5 U), which is shown by the broken line. The open circle indicates the patient who died 3 months after disease onset. (**B**) Titres of anti-DT antibodies in ELISA. The same serum samples as used in (**A**) were measured. Horizontal bars show the mean values of OD for the sera group in the active stage (except for the patient indicated by the open circle) and for the sera group in the inactive stage.



with anti-MDA-5 antibodies who experienced clinical remission for >5 years, we investigated anti-MDA-5 antibodies in these surviving patients. Our results showed that all but one patient lost anti-MDA-5 antibodies in sera and went into remission.

Kuwana *et al.* [13] examined serial changes in anti-topo I antibody levels in patients with SSc and found that, in some patients with a favourable outcome, loss of anti-topo I antibodies occurred within 10 years after the first visit. Kinetic studies of *in vitro* T-cell proliferation indicated that the disappearance of anti-topo I antibodies was due to loss of activation of topo I-reactive T cells. Expressions of cryptic epitopes by protein cleavage are probably important for the autoantibody response. MDA-5, which plays important roles in the innate immune system during RNA viral infections, is degraded in cells infected with different picornaviruses [14]. Whether such cleavage might lead to autoimmune responses against MDA-5 needs further investigation.

In summary, we have identified the disappearance of anti-MDA-5 antibodies in ADM remission. The precise factors or mechanisms that define positive/negative immune response to MDA-5 among ADM patients remain unknown. Future studies should address whether anti-MDA-5 antibody levels are useful as indicators for response to therapy. To confirm anti-MDA-5 antibodies as a marker for increased disease activity, future studies would need to determine whether anti-MDA-5 antibodies reappear during disease activity.

Rheumatology key messages

- Anti-MDA-5 antibodies could be an important serological marker for ILD in ADM patients.
- The tracking of anti-MDA-5 antibodies could be useful for monitoring disease activity in ILD complicated with ADM.

Acknowledgements

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Disclosure statement: The authors have declared no conflicts of interest.

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Novel adenosine triphosphate (ATP)-binding cassette, subfamily A, member 12 (*ABCA12*) mutations associated with congenital ichthyosiform erythroderma

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MADAM, Autosomal recessive congenital ichthyosis (ARCI) is a keratinization disorder, characterized by general desquamation. ARCI is a heterogeneous entity, including harlequin ichthyosis (HI, MIM 242500), lamellar ichthyosis type 2 (LI2, MIM 601277) and congenital ichthyosiform erythroderma (CIE, MIM 242100). The reported mutations in CIE include adenosine triphosphate (ATP)-binding cassette, subfamily A, member 12 (*ABCA12*),[1] transglutaminase 1 (*TGM1*),[2] lipoxygenase-3, 12(R)-lipoxygenase,[3] *NIPAL4*[4] and *CYP4F22*.[5] Mutations in *ABCA12* also result in LI2 and HI.[6,7] We report *ABCA12* mutations in four unrelated Japanese patients with CIE and identified five unreported and two recurrent mutations.

Patient 1 is a 3-year-old girl with generalized scales on erythroderma, ectropion, eclabium, severely deformed ears and alopecia (Fig. 1a–c). Her elder sister displayed similar symptoms and died after dehydration and infection. Patient 2 is a 9-year-old girl with generalized scales on an erythrodermic skin, mild ectropion, alopecia of the forehead and mild auricular malformation. Her younger sister died after severe skin symptoms and subsequent complications. Patient 3 is a 4-month-old boy, born as a collodion baby, with systemic whitish scales and generalized erythrodermic skin. There is no family history. Patient 4 is a 3-month-old boy, born as a collodion baby, with generalized whitish scales on a mild erythrodermic skin (Fig. 1d,e). Ectropion, eclabium and auricular malformation were not seen. There is no family history. Pathological findings of all patients revealed hyperkeratosis, mild acanthosis and perivascular lymphocytic infiltration.

We initially examined for *ABCA12* mutation, because *ABCA12* mutations have been found frequently in Japanese patients with CIE. For analysis of the *ABCA12* gene, polymerase chain reaction (PCR) fragments were amplified with 53 primer pairs, as previously reported.[6] We identified five unreported and two recurrent mutations (Table 1). Patient 1 had compound heterozygosity of missense/small deletion mutations [(p.Thr1575Pro)+(c.6031delG)]. Patients 2 and 3 had compound heterozygosity of missense/splice-site mutations [(p.Arg986Trp)+(c.5940–1G>C), (p.Asn1380Ser)+(c.5128+3A>G), respectively]. Patient 4 had compound heterozygosity

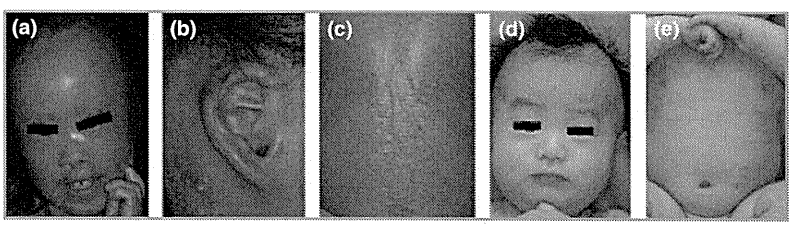

Fig 1. (a–c) Clinical features of patient 1. The whole body was covered with whitish scales on the erythrodermic skin. Ectropion, eclabium and alopecia of the forehead were seen. (d,e) Clinical features of patient 4. Whitish scales and generalized erythrodermic skin were seen.

Table 1 Summary of mutation analysis of *ABCA12* in the present study

Patient	Age, sex	Mutation	Maternal	Paternal
1	3 years, girl	Compound heterozygous	p.Thr1575Pro (c.4723A>C)	c.6031delG
2	9 years, girl	Compound heterozygous	p.Arg986Trp (c.2956C>T)	c.5940–1G>C
3	4 months, boy	Compound heterozygous	p.Asn1380Ser (c.4139A>G)	c.5128+3A>G
4	3 months, boy	Compound heterozygous	p.Thr1575Pro (c.4723A>C)	p.Gly1651Ser (c.4951G>A)

of missense mutations [(p.Thr1575Pro)+(p.Gly1651Ser)]. Each of the parents was a heterozygous carrier. Five mutations (p.Thr1575Pro, c.6031delG, p.Arg986Trp, c.5940–1G>C and c.5128+3A>G) have not been reported previously. Two recurrent mutations (p.Asn1380Ser and p.Gly1651Ser) have been reported previously in LI2.[6] These mutations were not found in 200 normal, unrelated Japanese alleles.

In cDNA from the skin of patient 2, reverse transcriptase-PCR (RT-PCR) across the c.5940–1G>C mutation site showed a single band of 526 bp. Subcloning and direct sequencing

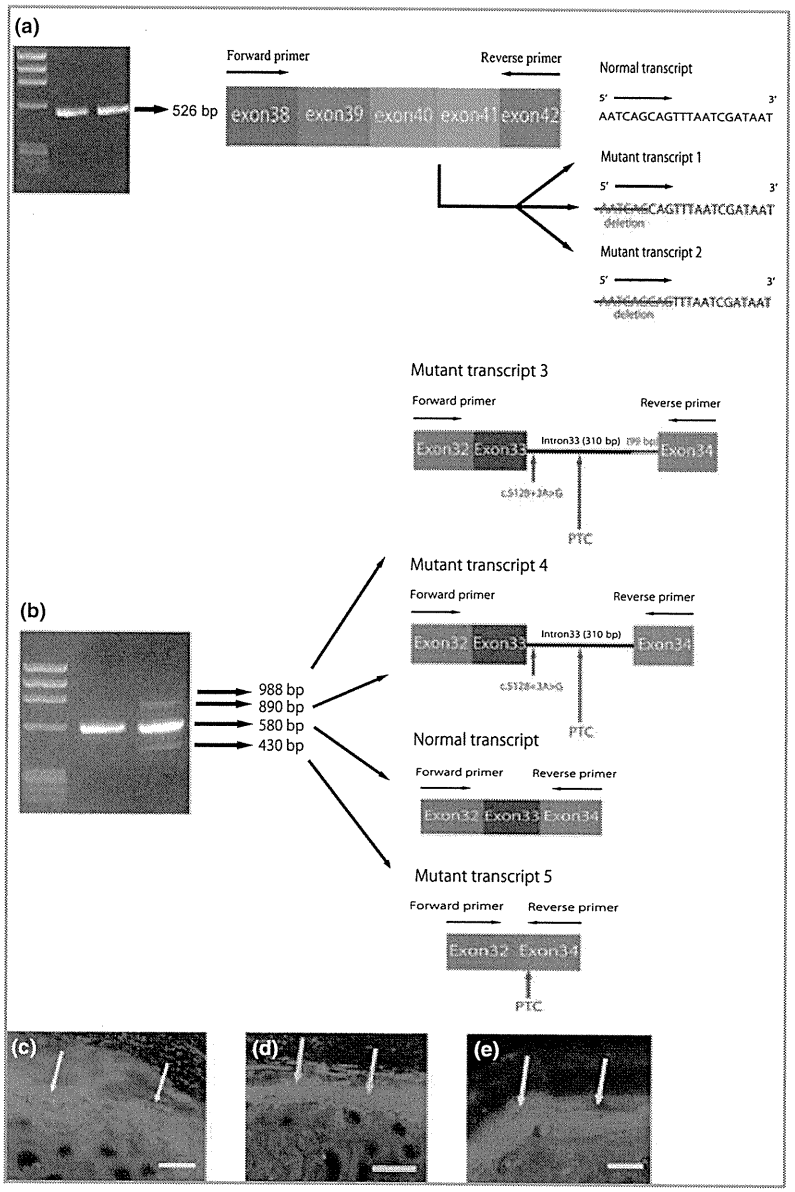

Fig 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mRNA fragments around the splice-site mutations and immunofluorescent analysis. (a) In patient 2, RT-PCR, subcloning and direct sequencing through the exon 40–41 boundary revealed two mutant transcripts as well as a normal transcript. Mutant transcript 1 had lost 6-bp nucleotides from exon 41, which resulted in a 2-amino acid deletion (Ile1981_Ser1982del). Mutant transcript 2 had lost 9-bp nucleotides from exon 41, which resulted in a 3-amino acid deletion (Ile1981_Ser1983del). Both mutant transcripts were within-frame deletions. (b) In patient 3, three aberrant mutant transcripts, all of which led to a premature termination codon, were identified by RT-PCR, subcloning and direct sequencing through the exon 33–34 boundary. Mutant transcript 3 was 988 bp in length with the inclusion of 310 bp and another 99 bp of intron 33. Mutant transcript 4 was 890 bp in length with the inclusion of 310 bp of intron 33. Mutant transcript 5 had exon 33 skipping. (c–e) Immunofluorescent labelling of ABCA12 in the skin. (c,d) A dot-like pattern of ABCA12 staining was seen in the cytoplasm of keratinocytes in the upper epidermis in patient 1 (c) and patient 2 (d). (e) In the normal control epidermis, ABCA12 staining was relatively strong in the granular layers and seemed to be dominant at the cell periphery. Bar = 5 μm.

revealed two mutant transcripts with in-frame deletions (Fig. 2a). In cDNA from the skin of patient 3, RT-PCR across the c.5128+3A>G mutation site identified four bands of 988, 890, 580 and 430 bp, with a single 580-bp band in the control sample (Fig. 2b). Subcloning and direct sequencing revealed three aberrant mutant transcripts, all of which led to premature termination codons. Immunofluorescence using anti-ABCA12 antibody revealed a diffuse staining of ABCA12 in the granular layers of control skin (Fig. 2e) and of the non-ABCA12 form (TGM1) from patient CIE (data not shown), while a dot-like staining in the cytoplasm was observed in patients 1 and 2 (Fig. 2c,d).

ABCA12 is a membrane lipid transporter that functions in the lipid transport from the trans-Golgi network to lamellar granules.[8] *ABCA12* mutations result in heterogeneity, including LI2, HI and CIE.[1,6,7] LI2 is characterized by generalized scales without serious erythroderma, and caused by either homozygote or compound heterozygote for missense mutations within the first nucleotide-binding folds of *ABCA12*.[6] HI is the severest form of ARCI, characterized by generalized large, plate-like scales with ectropion, eclabium and flattened ears.[7] HI is usually caused by homozygous or compound heterozygous truncation mutations in *ABCA12*.[7] In contrast, CIE with *ABCA12* mutation clinically shows milder manifestations.[1] Thus far, 17 different mutations in *ABCA12* have been reported in 12 cases of CIE. Eleven of 12 cases have at least one missense mutation. Only three of 17 mutations (p.Asn1380Ser, p.Ile1494Thr and p.Arg1514His) were located in the first nucleotide-binding folds. Other mutations were located outside ABCA12 active transporter sites: two nucleotide-binding folds and 12 transmembrane domains. The mutation p.Thr1575Pro was identified in two unrelated patients with different clinical severity. Patient 1 with severer features had a heterozygous truncation mutation (c.6031delG) on another allele, while patient 4, with a milder phenotype, had another heterozygous missense mutation (p.Gly1651Ser). We suggest that the phenotypic variability in these two patients was caused by different mutations.

We identified two *ABCA12* splice-site mutations, which were not reported in CIE: c.5128+3A>G and c.5940–1G>C. RT-PCR analysis across the site of the c.5940–1G>C mutation in patient 2 revealed two mutant transcripts. These findings demonstrate expression of the in-frame shorter transcript lacking two or three amino acids due to this splice-site mutation, which may account for the mild phenotype. In contrast, RT-PCR analysis across the site of the c.5128+3A>G mutation in patient 3 revealed three aberrant mutant transcripts, all of which led to premature termination codons. Therefore, patient 3 had a compound heterozygosity for missense/truncated combinations of mutations.

Using high-throughput sequencing analyses, screening of all ARCI-related genes is currently possible, but the cost is still expensive.[9] Once this is overcome, the elucidation of the pathogenesis of ARCI will greatly progress in the near future.

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Iatrogenic androgenetic alopecia in a male phenotype 46XX true hermaphrodite

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MADAM, Androgenetic alopecia (AGA) is a term that describes the androgen-dependent and genetically determined nature of the disease.[1] However, although it is known that androgen replacement therapy can induce AGA, no report has previously been issued regarding the development of iatrogenic AGA in a hermaphrodite undergoing androgen therapy. Herein, we describe a unique case of a castrated male phenotype 46XX true hermaphrodite receiving exogenous androgen supplementation who developed male-type hair loss.

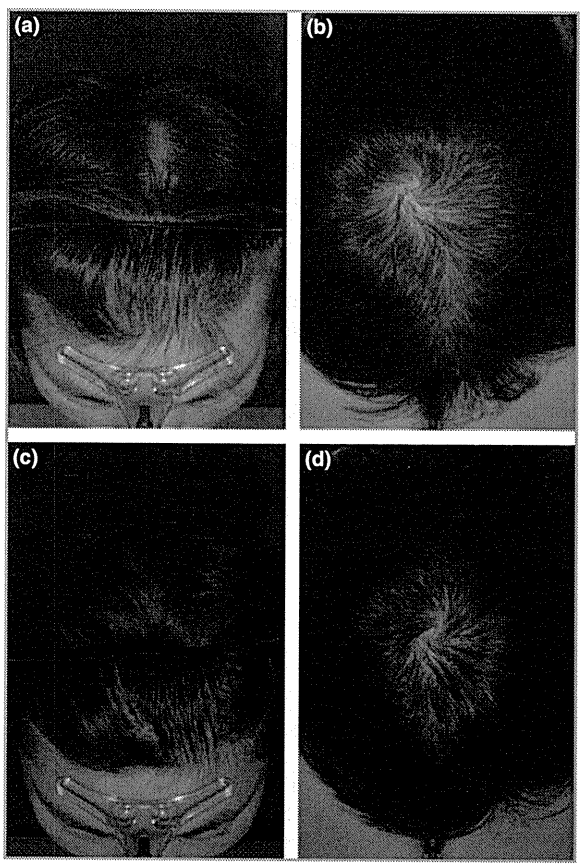

Fig 1. Iatrogenic androgenetic alopecia in a male phenotype 46XX true hermaphrodite showed a great improvement compared with baseline (a, b) after 4 months of finasteride treatment (c, d).

A 21-year-old male phenotype 46XX true hermaphrodite presented with a 3-year history of progressive hair loss. At the age of 16 years he was diagnosed as a 46XX true hermaphrodite with bilateral ovotestis, and subsequently underwent bilateral orchiectomy and testis prosthesis insertion. In addition, he was then given testosterone replacement therapy (testosterone enanthate, Jenasteron®; Jenapharm, Jena, Germany) for surgically induced andropausal status, which halted the development of secondary sexual characteristics. After 3 years of androgen therapy, progressive hair thinning developed on the scalp. Hair examination revealed nonscarring Norwood–Hamilton type III vertex alopecia with frontotemporal recession or BASP classification M1V2 alopecia (Fig. 1a, b).[2] Digital microscopy (Folliscope®; LeadM Corporation, Seoul, Korea) showed miniaturized hair shafts, and hair shaft size variation over the vertex scalp (Fig. 2). Serum testosterone, at the time, was 4·1 ng mL^{-1} (normal 2·7–10·7) and serum dehydroepiandrosterone sulphate was 1845 ng mL^{-1} (normal 800–5600). Under a diagnosis of iatrogenic androgen-induced alopecia, finasteride (1 mg daily) therapy was started. After 4 months of treatment, the hair loss stabilized and scalp hair regrowth was observed, despite the continuance of testosterone replacement therapy (Fig. 1c, d).

True hermaphroditism is an extremely rare disorder, which is defined as the coexistence of testicular and ovarian tissue in the same subject. The most frequent karyotype of true hermaphrodites is 46XX.[3] Gender assignments for hermaphrodites are made according to genetic, gonadal, social and psychologically determined sex, and the requests of patients and their relatives.[4] To be reared as male or female, surgical correction of ambiguous external genitalia, surgical removal of dysgenetic gonads, and sex hormone replacement for the surgically induced andropausal or menopausal state are required. The unwanted dermatological side-effects of testosterone replacement therapy include acne, excessive hair growth and male pattern baldness. As in our case, to be reared

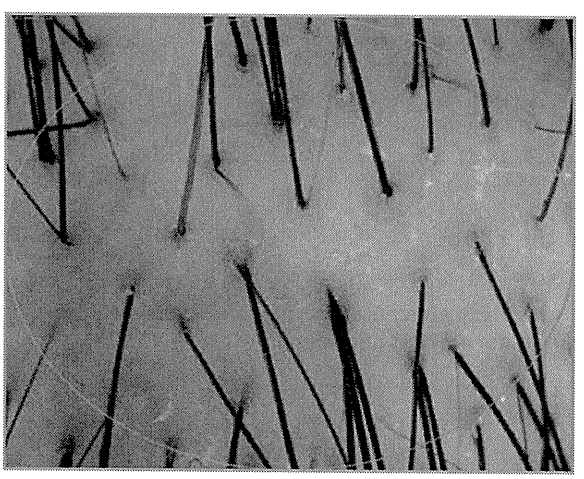

Fig 2. Photomicrograph showing miniaturized hair shafts, and variations in hair shaft size over the vertex scalp (original magnification ×50).

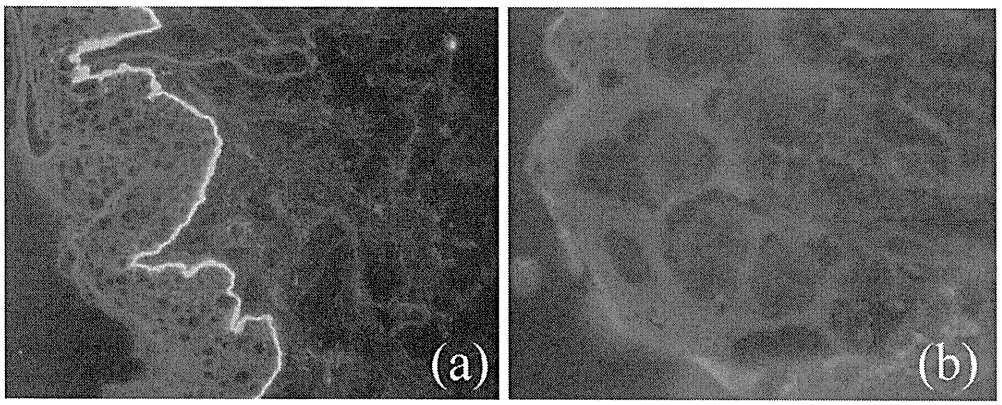

Fig. 2. Indirect immunofluoresence for collagen VII autoantibodies on normal skin (a) and collagen VII deficient skin (b) with serum from EBA patient, 200×.

We agree with the authors that more studies are indicated to determine the use of this test for monitoring disease activity in EBA patients. Similar studies in pemphigus patients with recombinant desmoglein 1 and 3 ELISA's reveal that the sera with identical titers of antibodies by IIF give variable results with ELISA [7]. Unless high titer sera are diluted, saturation of antibody–antigen reactions in ELISA may lead to false low positive ELISA index values to begin with. Such sera may not appear to show a decline in ELISA index values with treatment response [8]. We also have observed, in some pemphigus sera, that even though the IIF titers show a decline, ELISA index values still remain high. Therefore, we may have to use this ELISA with caution to monitor the disease.

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Letter to the Editor

CYP4F22 is highly expressed at the site and timing of onset of keratinization during skin development

Keywords:
Ichthyosis;
Keratinization;
Skin barrier

Autosomal recessive congenital ichthyoses (ARCI) include several subtypes: harlequin ichthyosis (HI), lamellar ichthyosis (LI) and congenital ichthyosiform erythroderma (CIE). To date, six causative genes have been identified in ARCI patients: *ABCA12*, *TGM1*, *NIPAL4*, *CYP4F22*, *ALOXE3* and *ALOX12B* [1]. The localization of transglutaminase 1, ABCA12 and 12R-lipoxygenase have been analyzed using samples from patients and model mice [1]. However, as for NIPAL4, CYP4F22, and lipoxygenase-3, neither localization nor function has been fully clarified yet. Herein, we investigate the expression pattern and localization of NIPAL4, CYP4F22 and lipoxygenase-3 in developing human epidermis and primary cultured normal human keratinocytes.

By quantitative reverse transcription (RT)-PCR analysis, at 10 and 14 weeks EGA, mRNA of *NIPAL4*, *CYP4F22* and *ALOXE3* was hardly expressed (Fig. 1A). The *CYP4F22* mRNA expression at 18

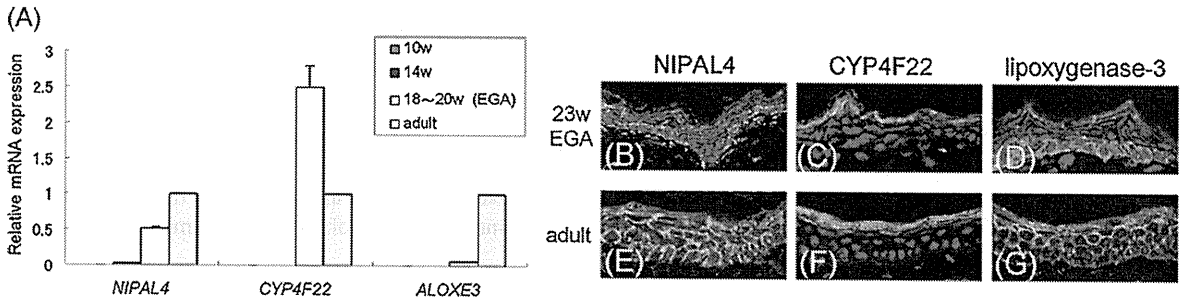

Fig. 1. NIPAL4, CYP4F22 and lipoxygenase-3 expression in developing human skin. (A) mRNA expression in developing human skin. The mRNA expression of *NIPAL4*, *CYP4F22* and *ALOXE3* in fetal human whole skin was studied by quantitative RT-PCR analysis, normalized by *GAPDH* [Applied Biosystems: Hs00398027_m1*, Hs00403446_m1*, Hs00222134_m1*, Hs03929097_ gl*]. At 10 and 14 weeks EGA, *NIPAL4*, *CYP4F22* and *ALOXE3* mRNA are hardly expressed. At 18–20 weeks EGA, the rate of *CYP4F22* mRNA expression is higher than in adult human whole skin ($n = 3$, mean ± SD). (B–G) Immunofluorescence staining of NIPAL4, CYP4F22 and lipoxygenase-3 in developing human skin. Fetal skin samples at 10–23 weeks EGA and adult skin samples were stained for NIPAL4 [Rabbit polyclonal anti-NIPAL4 antibody against a 16-amino acid sequence synthetic peptide (residues 445–461)], CYP4F22 [B01; Abnova, Taipei City, Taiwan], and lipoxygenase-3 [T-14; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.] (Supplementary Fig. S1). For the 23 weeks EGA sample and the adult skin, CYP4F22 (C and F) is expressed in the upper layer of the epidermis, mainly in the granular layers. NIPAL4 (B and E) and lipoxygenase-3 (D and G) are expressed at the cell periphery throughout the epidermis. NIPAL4 expression is seen evenly from the basal cell layer to the granular layers, although lipoxygenase-3 expression is slightly stronger towards the granular layers. NIPAL4, CYP4F22 and lipoxygenase-3 green (FITC), nuclear stain, red (PI solution) (original magnification 40×). Data are presented as representative of triplicate experiments.

and 20 weeks EGA was higher than that in adult human skin. At 18 and 20 weeks EGA, *NIPAL4* mRNA expression was approximately half of that in adult skin, and only a tiny amount of *ALOXE3* mRNA was expressed.

We investigated protein localization by immunofluorescence staining (Fig. 1B–G). For the 10 weeks EGA sample, NIPAL4, CYP4F22 and lipoxygenase-3 were not detected. A similar pattern was obtained for the 14 weeks EGA sample. For the 23 weeks EGA sample, CYP4F22 was expressed in the upper layer of epidermis, mainly in the granular layers, and NIPAL4 and lipoxygenase-3 were expressed at the cell periphery in the entire epidermis. Staining patterns of NIPAL4, CYP4F22 and lipoxygenase-3 in the adult skin were similar to those at 23 weeks EGA. Lipoxygenase-3 is usually considered to be a partner with 12R-LOX. 12R-LOX has been visualized at the cell periphery only in the upper epidermis [2]. In our results, lipoxygenase-3 was distributed at the cell periphery in the entire epidermis. Concerning to lipoxygenase-3 in the upper epidermis, lipoxygenase-3 is thought to work with 12R-LOX, although function of lipoxygenase-3 in the lower epidermis is unknown.

In cultured keratinocytes, RT-PCR analysis (Fig. 2A) and immunoblot analysis (Fig. 2B and C) confirmed that mRNA and protein expression of CYP4F22 were increased under the high Ca^{2+} condition (1.2 mmol/L for 48 h). In contrast, there was no significant increase in the mRNA or protein expression of NIPAL4 or ALOXE3 under the high Ca^{2+} condition.

The present study of the adult human epidermis clarified that NIPAL4 and lipoxygenase-3 were expressed at the cell periphery in the entire epidermis of adult human skin. CYP4F22 was expressed in the cytoplasm of keratinocytes in the upper layer of adult human epidermis, mainly in the granular layers. One previous report [3] noted that, inconsistent with our present observations, *NIPAL4* mRNA is highly expressed in the granular layers of the epidermis with *in situ* hybridization analysis. The cause of this discrepancy is unclear, but it might be due to difference in sensitivity between *in situ* hybridization and immunostaining.

We have demonstrated that the mRNAs of *NIPAL4*, *CYP4F22* and *ALOXE3* are not expressed in the early stages of fetal development, at 10 weeks EGA or at 14 weeks EGA. At 18 and 20 weeks EGA, *NIPAL4* mRNA expression was about half that in adult skin, although *ALOXE3* mRNA was only weakly expressed. Among the keratinization-associated genes, the mRNA expression pattern of *NIPAL4* is similar to that of *ABCA12*, and the pattern of *ALOXE3* resembles those of other keratinization-related molecules, such as *TGM1*, *LOR* and *KLK7* [4].

NIPAL4 encodes a putative transmembrane protein of 404 amino acids with a molecular weight of 44 kDa [6]. The NIPAL4 protein is highly expressed in the brain, lung and stomach, and in

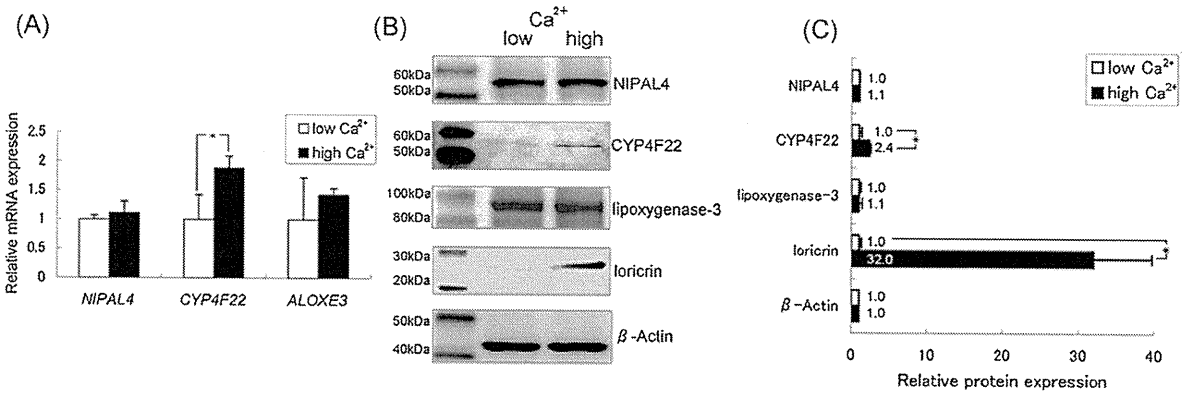

Fig. 2. mRNA and protein expression of NIPAL4, CYP4F22 and ALOXE3 in developing human skin and NHEK. (A) mRNA expression in NHEK. mRNA expression of *CYP4F22* is significantly higher in the NHEK under the high Ca^{2+} condition than in those under the low Ca^{2+} condition. There are no significant differences between the high and low Ca^{2+} conditions in terms of the mRNA expression of *NIPAL4* and *ALOXE3* ($n = 3$, mean ± SD, $^*p < 0.05$). (B) Protein expression assessed by Western blot analysis. The expression of CYP4F22 is higher in the NHEK raised under the high Ca^{2+} condition than in those raised under the low Ca^{2+} condition. However, neither NIPAL4 nor lipoxygenase-3 is increased under high Ca^{2+} condition. Anti-ALOXE3 antibody for immunoblotting: NBP1-32533; Novus Biologicals, LLC, U.S.A. (C) Quantitative analysis by Image J software revealed that the protein expression of CYP4F22 was significantly increased under the high Ca^{2+} condition. Data are presented as representative of triplicate experiments.

leukocytes and keratinocytes. The protein product of the *ALOXE3* gene, lipoxygenase-3, is thought to function as a hydroperoxide isomerase to generate epoxy alcohol [5]. CYP4F22 is a member of the cytochrome P450 family 4, subfamily F. The gene includes 12 coding exons and the cDNA spans 2.6 kb in length. All *CYP4F22* mutations reported to date are predicted to abolish the function of the encoded CYP protein and to compromise the 12(R)-lipoxygenase (hepoxilin) pathway.

Human epidermis contains 15S-lipoxygenase type 1, 12S-lipoxygenase and 12R-lipoxygenase [6]. Skin also contains cytochrome 450, and members of the CYP4 family with unknown epidermal function [3]. 12R-lipoxygenase has attracted great medical interest. 12R-lipoxygenase is expressed only in the epidermis and the tonsils [6,7] and is upregulated in psoriatic lesions [8]. It transforms 20:4n-6 to 12R-hydroperoxyeicosatetraenoic acid (12R-HPETE), which is important for the development of the water permeability barrier function in the epidermis [2]. 12R-LOX and eLOX3 play a crucial role in releasing ω-hydroxyceramide for construction of the corneocyte lipid envelope which is essential for intact skin barrier [9]. O-linoleoyl-ω-hydroxyceramide is oxygenated by the consecutive actions of 12R-LOX and eLOX3 and the products are covalently attached to protein *via* the free ω-hydroxyl of the ceramide, forming the corneocyte lipid envelope [9].

It is hypothesized that CYP4F22 may be linked to the 12R-lipoxygenase and lipoxygenase-3 pathway. Hydroxyeicosatetraenoic acids (HEETs) can be hydrolyzed to triols by epoxide hydrolases, and these products might be substrates of CYP4F members. Thus, it is possible that CYP4F22 might be involved in a downstream step in the 12R-lipoxygenase/lipoxygenase-3 pathway. CYP4F22 could be involved in the oxidation of 8R,11R,12R-HEET. However, from a systemic study of MS/MS spectra of HEETs derived from 12- and 15-HPETE, CYP4F22 did not appear to oxidize 8R,11R,12R-HEET [10]. Nilsson et al. [10] reported that recombinant CYP4F22 catalyzed the omega-3 hydroxylation of 20:4n-6; however, oxygenation of 8R,11R,12R-HEET was not detected. An additional function of CYP4F22 is to synthesize the omega-hydroxy fatty acids in the ceramide [10].

Our study revealed CYP4F22 to be highly expressed at the site and the onset of keratinization during skin development. From this it is speculated that CYP4F22 is involved in the metabolism of lipid substrates that are important to differentiation/keratinization of epidermal keratinocytes, at least during the fetal period. Further studies of the function of CYP4F22 would be needed to elucidate its function in development of the epidermis and keratinocytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jdermsci.2011.12.006.

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ORIGINAL ARTICLE

Applicability of radiocolloids, blue dyes and fluorescent indocyanine green to sentinel node biopsy in melanoma

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ABSTRACT

Patients with primary cutaneous melanoma underwent sentinel node (SN) mapping and biopsy at 25 facilities in Japan by the combination of radiocolloid with gamma probe and dye. Technetium-99*m* (^{99m}Tc)-tin colloid, ^{99m}Tc-phytate, 2% patent blue violet (PBV) and 0.4% indigo carmine were used as tracers. In some hospitals, 0.5% fluorescent indocyanine green, which allows visualization of the SN with an infrared camera, was concomitantly used and examined. A total of 673 patients were enrolled, and 562 cases were eligible. The detection rates of SN were 95.5% (147/154) with the combination of tin colloid and PBV, 98.9% (368/372) with the combination of phytate and PBV, and 97.2% (35/36) with the combination of tin colloid or phytate and indigo carmine. SN was not detected in 12 cases by the combination method, and the primary tumor was in the head and neck in six of those 12 cases. In eight of 526 cases (1.5%), SN was detected by PBV but not by radiocolloid. There were 13 cases (2.5%) in which SN was detected by radiocolloid but not by PBV. In 18 of 36 cases (50%), SN was detected by radiocolloid but not by indigo carmine. Concomitantly used fluorescent indocyanine green detected SN in all of 67 cases. Interference with transcutaneous oximetry by PVB was observed in some cases, although it caused no clinical trouble. Allergic reactions were not reported with any of the tracers. ^{99m}Tc-tin colloid, ^{99m}Tc-phytate, PBV and indocyanine green are useful tracers for SN mapping.

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Key words: indocyanine green, melanoma, patent blue violet, phytate, sentinel lymph node biopsy, tin colloid.

INTRODUCTION

Intraoperative lymphatic mapping and selected lymphadenectomy have made it possible to determine the lymphatic flow from a primary tumor and to identify its sentinel lymph node(s) (SN) in the regional basin.[1–3] Since its sixth edition, the American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) melanoma staging system has incorporated the pathological evaluation in SN into the new staging criteria for melanoma.[4] In Japan, sentinel node biopsy (SNB) has been verified for over a decade and is now widely performed in melanoma staging.[5,6] However, prior to the completion of the present study, SNB had been performed only in the context of clinical trials and had not been covered by public health insurance in Japan. In order to gain approval for clinical use and to gain insurance coverage, we planned a multicenter clinical trial under the unified methods.

METHODS

The multicenter study was performed from January 2007 to October 2009. Patients underwent cutaneous lymphoscintigraphy with radiopharmaceuticals, either 1 day before or on the day of the operative procedure. ^{99m}Tc-tin colloid and ^{99m}Tc-phytate, which have particle sizes ranging 400–5000 nm and 200–1000 nm, respectively, were used as radiocolloids. Using the lymphoscintigraphy, the skin site corresponding to the highest accumulation of radioparticles was identified and marked. At the time of surgery, 2% patent blue violet or 0.4% indigo carmine was injected i.d. at the primary tumor site. Furthermore, in some hospitals, 0.5% fluorescent indocyanine green, which allows visualization of the SN with an infrared camera, was concomitantly used in the combination of radiocolloid and blue dye. An incision was made on the skin site that was the most radioactive by the gamma probe.

Candidates for the study were patients with primary invasive cutaneous melanoma without any clinical evidence of metastatic disease. Exclusion criteria were as follows: patients with distant metastasis, patients with severe liver dysfunction, patients who were nursing infants or who were pregnant or might be pregnant, patients who did not provide informed consent and patients regarded as not suitable for this study by their physicians. Before this study, the protocol of SNB was approved by the ethics committee of each institution and was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR: www.umin.ac.jp/ctr/index-j.htm).

RESULTS

A total of 673 patients were enrolled. Each patient who had received SN mapping with a combination of blue dye and radiocolloid with a handheld gamma probe were eligible for analysis of the detection rate; 562 patients met that criterion. The detection rate of SN in those cases was 97.9% (550/562). The detection rates of SN were 95.5% (147/154) in patients given the combination of tin colloid and patent blue violet, 98.9% (368/372) in those given the combination of phytate and patent blue violet, and 97.2% (35/36) in those given the combination of tin colloid or phytate and indigo carmine (Table 1). SN was not detected in 12 cases by the combination method. In six of those cases, the primary tumor was in the head and neck (Table 2). In eight of 526 cases (1.5%), SN was detected by patent blue violet but not by tin colloid (seven cases) or by

Table 1. Tracers and detection rates (the combination of dye and radiocolloid with gamma probe)

Tracer	Detection rate	No. of cases
PB and phytate	98.9% (368/372)	372
PB+, phytate+		366
PB+, phytate–		1
PB–, phytate+		1
Not detected		4
PB and tin-colloid	95.5% (147/154)	154
PB+, tin-colloid+		127
PB+, tin-colloid–		7
PB–, tin-colloid+		13
Not detected		7
I and phytate/tin-colloid	97.2% (35/36)	36
I+, phytate or tin-colloid+		17
I+, phytate or tin-colloid–		0
I–, phytate or tin-colloid+		18
Not detected		1

+, detected; –, not detected; I, indigo carmine; PB, patent blue violet.

Table 2. Profiles of the cases in which SN was not detected (n = 12)

Location (no. of cases)	Radiocolloid	Dye
Head and neck (n = 6)		
Cheek	T	PB
Ear	P	PB
Ear	T	I
Eyelid	T	PB
Cheek	T	PB
Lip	T	PB
Trunk (n = 2)		
Chest	T	PB
Back	T	PB
Upper extremity (n = 2)		
Upper arm	T	PB
Finger	P	PB
Lower extremity (n = 2)		
Sole	P	PB
Sole	P	PB

I, indigo carmine; P, phytate; PB, patent blue; SN, sentinel node; T, tin colloid.

phytate (one case). There were 13 cases (2.5%) in which SN was detected by radiocolloid but not by patent blue violet. In 18 of 36 cases (50%), SN was detected by radiocolloid but not by indigo carmine. Concomitantly used, fluorescent indocyanine green detected SN in all 67 cases. Toxicity was evaluated in 673 cases. Interference with transcutaneous oximetry by patent blue violet was observed in some cases, although it caused no clinical trouble. Allergic reactions were not reported in tin colloid (154 cases), phytate (372 cases), patent blue violet (527 cases), indigo carmine (36 cases) or indocyanine green (94 cases). No other problems related to the tracers were reported.

DISCUSSION

Our results showed that both ^{99m}Tc-tin colloid and ^{99m}Tc-phytate detected SN with a high degree of accuracy. The SN detection rates were 95.5% with the combination of tin colloid and patent blue violet and 98.9% with the combination of patent blue violet and phytate. In six of 12 cases in which SN was not detected, the primary tumor was in the head and neck region, similar to the tendencies in previous reports.[7] In eight cases, SN was detected by patent blue violet but not by tin colloid (seven cases) or by phytate (one case). We supposed that halation may have caused the failure in four of seven cases in which tin colloid was used, because of the closeness of the primary tumor and SN. In the 527 cases in which patent blue was used, there were 13 cases in which SN was detected by radiocolloid but not by patent blue violet. Technical factors may have influenced the phenomenon. Indigo carmine could not detect SN in half of the cases. The concentration might be too low to detect SN, because the approved concentration of indigo carmine in Japan is now only 0.4%. These results indicate that the combination of radio colloid and dye is important for detecting SN without failure.

Concomitantly used, fluorescent indocyanine green detected SN in all 67 cases. Because indocyanine green has a characteristic absorption peak of 800 nm in the near-infrared region, it can be used for real-time fluorescence navigation to detect SN.[8] This method might be useful as a complement to lymphoscintigraphy, the gamma probe technique and blue dye.

Reported adverse effects of isosulfan and patent blue dyes include allergic reactions such as urticaria, rash and, rarely, anaphylaxis, protracted blue discoloration, interference with transcutaneous oximetry and cutaneous necrosis.[3] In our study, interference with transcutaneous oximetry by patent blue violet was observed in some cases, although it caused no clinical trouble. No allergic reactions were observed.

In conclusion, radiocolloids, blue dyes and indocyanine green were useful tracers for SN mapping in this study. The concentration of indigo carmine might need to be substantially increased. These data were sent to the Ministry of Health, Labor and Welfare of Japan. In April 2010, SNB was approved by the Japanese public health insurance system.

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INVESTIGATIVE REPORT

Limited Influence of Aspirin Intake on Mast Cell Activation in Patients with Food-dependent Exercise-induced Anaphylaxis: Comparison Using Skin Prick and Histamine Release Tests

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Food-dependent exercise-induced anaphylaxis (FDEIA) is a severe systemic syndrome induced by physical exercise after ingesting causative food. Aspirin is a well-known trigger for anaphylaxis in patients with FDEIA. Possible mechanisms by which symptoms are aggravated by aspirin include enhanced antigen absorption and mast cell activation. The aim of this study was to determine whether aspirin intake has an influence on mast cell/basophil activation in patients with FDEIA. Provocation tests revealed that adding aspirin to the causative food challenge in 7 of 9 (77.8%) patients with FDEIA provoked symptoms. In most cases, pretreatment with aspirin did not enhance skin tests (71.4%) or histamine release tests (88.9%) with food allergen challenges. The study confirmes that histamine release and skin prick tests can be adjunctive tools for diagnosing FDEIA. In addition, our results suggest that exacerbation of FDEIA symptoms by aspirin is not mediated by direct effects of aspirin on mast cell/basophil activation. ***Key words: food-dependent exercise-induced anaphylaxis; aspirin; histamine release test; skin test; mast cells; basophils.***

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Food-dependent exercise-induced anaphylaxis (FDEIA) is a unique form of food allergy induced by exercise after food ingestion (1–4). Although the symptoms of FDEIA and cholinergic urticaria occur after exercise, FDEIA is clearly distinguishable from cholinergic urticaria, which appears as pinpoint-sized wheals with surrounding erythema that occur after sweating (5, 6). Patients with FDEIA usually have immunoglobulin E (IgE) antibodies and positive skin prick tests (SPTs) against the causative food allergens. Thus, the reactions in patients with FDEIA are generally thought to be IgE-mediated hypersensitivity to food allergens (7–10).

The causative foods are mainly wheat products and shellfish in Japan (11) and vegetables in European countries (8). Triggering factors for FDEIA include foods, exercise (12), the patient's general condition, the menstrual cycle (9, 13), and medications such as aspirin and non-steroidal anti-inflammatory drugs (14).

In particular, aspirin has been well demonstrated to induce or aggravate symptoms in patients with FDEIA (10, 15, 16). Several studies have reported that the SPT reaction to causative food allergens is enhanced by pretreatment with oral aspirin, which suggests that aspirin accelerates histamine release from mast cells and basophils, manifesting as an immediate hypersensitivity reaction (15, 17, 18). In contrast, another study demonstrated that, in patients with wheat-dependent exercise-induced anaphylaxis, aspirin uptake induced a marked increase in serum gliadin levels with the accompanying symptoms, which suggests that aspirin may up-regulate antigen uptake across the intestinal epithelium (16, 19). these two mechanisms have been proposed to account for the induction or exacerbation of symptoms by aspirin in patients with FDEIA. The aim of this study was to determine whether aspirin intake has an influence on mast cell/basophil activation in patients with FDEIA.

We performed skin tests and histamine release (from basophils) tests in 10 patients diagnosed with FDEIA, exposing them to causative food allergens by means of provocation tests before and after aspirin uptake. Neither test showed significant differences before or after aspirin uptake in most of the patients with FDEIA. These data support the opinion that aspirin intake enhances antigen uptake across the intestinal epithelium, but not activation of mast cells/basophils in most types of FDEIA.

MATERIALS AND METHODS

Patients

Six female and four male patients (ages 17–72 years) with FDEIA were enrolled in the study (Table I). All patients had a clinical history of exercise-induced anaphylaxis, especially with prior food intake. Two of the patients had allergic rhinitis. Oral and written informed consent for the study was obtained from all subjects.

Relevant drugs, such as histamine H_1-receptor antagonists, were withdrawn for at least 48 h before the trials. FDEIA was confirmed by provocation tests with the suspected foods,

Table I. *Clinical features of patients with food-dependent exercise-induced anaphylaxis*

Pat. no.	Age, years/sex	Responsible food	Total IgE IU/ml	Symptoms	Atopic diseases
1	66/F	Wheat	134	U, S	None
2	17/M	Spinach	634	U, D	AR
3	45/F	Wheat	75	U, S, A	None
4	34/F	Wheat	341	U, S	None
5	56/M	Wheat	126	U, S	None
6	53/M	Wheat	2,071	U, S	None
7	32/F	Wheat	37	U, D, A	None
8	17/F	Shrimp	991	U, D, A	AR
9	57/M	Shrimp	165	U, S	None
10	42/F	Wheat	450	U, S, D, A	None

U: urticaria; S: shock; D: dyspnoea; A: angioedema; AR: allergic rhinitis.

providing a definitive diagnosis. Cholinergic urticaria and exercise-induced anaphylaxis were ruled out because of the lack of symptoms after exercise alone. The responsible foods in patients with FDEIA include wheat, shrimp, and spinach.

Provocation tests

Provocation tests included challenges with the suspected foods, exercise, aspirin intake, and combinations of these challenges. For the wheat challenge, one piece of bread made from wheat flour (66 g) and a small amount of salt, was prepared (16). For the shrimp challenge, one fried shrimp was allotted to each patient. For the spinach challenge, 100 g of boiled spinach was prepared. Treadmill exercise was performed for 30 min after food ingestion using a slightly modified form of the protocol described by Bruce et al. (20). Aspirin (500 mg) was administered just before food intake, except in patient 4, who presented with aspirin-intolerant urticaria. When urticaria or other symptoms appeared, an antihistamine or adrenalin was given immediately.

Skin testing

Skin prick testing was performed with commercial food extracts (1:10 w/v) (Torii Pharmaceutical, Tokyo, Japan), gluten, and gliadin (1:10 w/v) (Wako, Osaka, Japan), using prick lancets (AB Nordic Medifield, Stockholm, Sweden). Reactions were read as the longest length × the perpendicular length of the wheal at 15 min. The responses were then compared with those of the positive histamine controls (10 mg/ml) and were scored as follows: 0, no positive area compared with that of the wheal on the histamine-positive control; 1+, 25%; 2+, 50%; 3+, >100%. At 2 h after aspirin intake, the SPT was performed again by the same person. The reaction to the positive histamine control was not enhanced after aspirin intake, except in patient 8.

Histamine release tests and IgE measurement

Histamine release tests were performed *in vitro* using the histamine release test (HRT) (Shionogi, Osaka, Japan) as described previously (5). Briefly, 20 µl peripheral venous blood samples from the patients and anti-basophil antibodies (BA312) conjugated with magnetic beads were added to each well of 96-well plates. They were incubated for 10 min at room temperature on a plate mixer. Antibody-binding basophils in each well were then trapped with a chandelier-shaped magnet and transferred to another microplate, where the basophils were stimulated at 37°C for 1 h with a diluted extract of a food allergen (Torii Pharmaceutical), anti-IgE antibody (21), and digitonin, respectively. Histamine released into the medium was measured using an enzyme-linked immunosorbent assay (ELISA) with a characteristic detection profile. The mean values and standard deviation (mean ± SD; $n=3$) of the percent histamine release from basophils of patients with the respective dilutions of commercial food extracts are shown in Table SI (available at: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1210). The statistical significance of differences between means was determined using Student's *t*-test.

Blood samples for the HRT after aspirin intake were collected 2 h after aspirin administration. When the histamine release was more than 20%, it was deemed a positive reaction. Total IgE levels and specific IgE levels were determined by fluoroenzymeimmunoassay (FEIA) methods. The cut-off value for the CAP-FEIA was set at 0.35 U_A/ml. Values < 0.34 U_A/ml were recorded as class 0 (negative); class 1 was 0.35–0.69 U_A/ml; class 2 was 0.70–3.49 U_A/ml; class 3 was 3.50–17.49 U_A/ml; and class 4 was 17.50–49.99 U_A/ml. Classes 2, 3, and 4 were deemed positive.

RESULTS

Provocation tests

All patients had a clinical history of exercise-induced anaphylaxis, especially with prior food intake (Table I). Elevated total IgE (> 270 IU/ml) levels were observed in five of the 10 patients (Table I). The results of the challenge tests are shown in Table II. Neither intake of foods alone nor exercise alone induced symptoms in any of the patients. In patient 4, aspirin intake alone provoked urticaria, suggesting that this patient also had aspirin-intolerant urticaria (22). In 4 patients (numbers 1, 3, 4, 9), ingestion of food followed by exercising resulted in urticaria. In 3 patients (numbers 3, 5, 7), ingestion of food after aspirin intake provoked urticaria. In patients 2, 6, 8, and 10, the combination of food intake, aspirin, and exercise caused urticaria, angioedema, or dyspnoea, whereas the combinations of food and exercise or food and aspirin did not provoke symptoms. Thus, adding aspirin to the causative food challenge in seven of nine patients with FDEIA provoked urticarial or upper respiratory symptoms.

Table II. *Results of provocation tests for patients with food-dependent exercise-induced anaphylaxis after 15 min exercise*

Pat. no.	Food	Aspirin, mg	Symptoms: Aspirin alone	Food + Exercise[a]	Food + Aspirin	Food + Aspirin + Exercise
1	Bread	500	None	U	None	Not done
2	Spinach	500	None	None	None	U, A
3	Bread	500	None	U	A, D	Not done
4	Bread	100	U	U, P	Not done	Not done
5	Bread	500	None	None	U	Not done
6	Bread	500	None	None	None	U
7	Bread	500	None	None	U, E	Not done
8	Shrimp	500	None	None	None	U, D
9	Shrimp	500	None	U, E	None	Not done
10	Bread	500	None	None	None	U, P, A

[a]No provocation for food alone or exercise alone.
U: urticaria; A: angioedema; P: palpitation; E: erythema; D: dyspnoea.